



Regeneration of *Trachyspermum ammi* L. Sprague ecotypes via indirect somatic embryogenesis using hypocotyl and epicotyl explants

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Trachyspermum ammi L. Sprague, commonly known as ajwain, carom or thymol seeds, is one of the oldest medicinal plants with numerous health benefits. The classical breeding process of medicinal plants is not only time consuming but also requires much efforts. Biotechnological techniques such as *in vitro* regeneration which increase the capacity of production of medicinal plants rely upon a plenty of components. Here, we studied regeneration of *T. ammi* by indirect somatic embryogenesis by employing MS and B5 media supplemented with a diverse concentration of plant growth regulators (PGRs), including 2,4-D, kinetin, NAA, IAA, and BAP with four explants (hypocotyl, epicotyl, root and node) and 3 ecotypes (Qom, Yazd, and Rafsanjan). The highest callus induction was obtained from hypocotyl explants on MS medium supplemented with 8.917 $\mu\text{m/L}$ 2,4-D and 2.32 $\mu\text{m/L}$ KIN and epicotyl explants on B5 medium supplemented with 1.074 $\mu\text{m/L}$ NAA and 0.89 $\mu\text{m/L}$ BAP. The highest indirect regeneration (83%) was observed in hypocotyl explants on MS medium supplemented with 0.93 $\mu\text{m/L}$ kinetin. To the best of our knowledge, the present study (2017) is the first report on indirect somatic embryogenesis from hypocotyl and epicotyl of the Iranian *Trachyspermum ammi* ecotypes that can be beneficial for genetic transformation and other plant biotechnology techniques.

Keywords: Ajwain, Callus induction, Carom, Herbal, Plant growth regulators, Thymol seeds, Traditional medicine

Trachyspermum ammi L. Sprague, commonly called Ajwain, Carom or Thymol seeds, is a traditional medicinal herb, which is highly effective in curing various human and animal diseases. It grows wild in different parts of Iran, India, Pakistan and Egypt¹. Some of the therapeutic characteristics of ajwain are treat gout and rheumatism², activate immune response and production of antibodies³, cure stomachache and Antihyperlipidaemic⁴. *Trachyspermum ammi* has many therapeutic applications in Persian traditional medicine⁵. Ajwain seed contains 2-4% essential oil, the most important component of which is thymol⁶. Thymol has many medical uses, including curing gastrointestinal problems, respiratory problems and lack of appetite⁷.

Medicinal herbs produce valuable secondary metabolites, with many uses like pharmaceuticals, healthcare industry, food flavors, perfumes, insecticides, etc. The production of these compounds is associated with many problems due to low production, non-uniformity of quality, and natural conditions. However, applying biotechnological

methods for selection, propagation and maintenance of medicinal plants is essential⁸. In recent years, employing tissue culture techniques as a quick and effective method to preserve valuable medicinal plants and produce secondary metabolites has been dramatically and rapidly rising. Nowadays, somatic embryos are widely used in biochemical, physiological, morphological and genetic research studies. These embryos are obtained from single cells and are able to be regenerated, making them suitable for storage of plant germplasm⁹. Somatic embryogenesis involves several *in-vitro* phases of initiation, growth, and germination of somatic embryos¹⁰. Auxin and cytokinin growth regulators have a significant role in growth quality in explants. In former researches, the efficacy of auxins and cytokinins for regeneration of several medicinal plants has been studied¹¹⁻¹³. To transfer proper genes to medicinal plants via *Agrobacterium* and other methods, an appropriate tissue culture system is necessary for plant regeneration from a transgenic cell. Regeneration of a large number of transgenic plants with foreign gene plays a vital role in improving their traits¹⁴. The most important step for *in vitro* regeneration is selecting the proper explant.

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Hypocotyl and epicotyl as appropriate explants have been reported in the regeneration of some medicinal plants in previous studies¹⁵⁻²³.

Koca & Asim²⁴ published a paper in which they described direct shoot regeneration from seed explants of *Trachyspermum ammi*. Salehi *et al.*²⁵ showed that direct shoot and root regeneration of *T. ammi* using apical bud explants on MS medium supplemented with BAP (4.4 µm/L) and IAA (0.5 µm/L). Purohit & Kothari⁷ reported that direct somatic embryogenesis occurred in MS medium containing 2,4-D (0.2 mg/L) from cotyledon and cotyledonary node explants of *T. ammi*.

In the present study, we tried to optimize the indirect regeneration of the Iranian ecotypes of *Trachyspermum ammi* (Qom, Yazd and Rafsanjan), using hypocotyl and epicotyl explants on MS and B5 media supplemented with various concentrations of plant regulators. This method can be efficacious and beneficial in gene transfer, propagation and other uses in plant biotechnology.

Materials and Methods

Seed sterilization and explant preparation

Trachyspermum ammi seeds of three ecotypes of Qom, Rafsanjan, and Yazd were obtained from Research Institute of Forest and Rangelands of Iran and authenticated at the Department of Agronomy and Plant breeding, College of Aburaihan, University of Tehran. For the purpose of seed sterilization, the seeds were surface-sterilized with 70% ethyl alcohol for 1.0 min and then treated immediately by 1.5% sodium hypochlorite solution for 7 min. Subsequently, they were rinsed with sterile water three times. After drying on sterile filter paper for 15 min, the seeds were cultured directly in a tube containing MS²³ and B5²⁴ media. The tubes were transferred to the growth chamber with 16 h of photoperiod and maintained at 25±2°C.

Establishment of seedling culture and callus induction

Twenty-day-old seedlings were utilized for tissue culture. This experiment was carried out by 3 ecotypes and 4 explants. One-cm-long hypocotyl, epicotyl, root, and node explants were cultured on MS and B5 media supplemented with different concentrations of plant growth regulators (PGRs) based on following ratios : (1) MS + 8.92 µm/L 2,4-D + 2.32 µm/L kin; (2) MS + 4.5 µm/L 2,4-D + 0.5 µm/L kin; (3) B5 + 1.07 µm/L NAA + 0.89 µm/L BAP; and (4) B5 + 1.07 µm/L NAA + 0.45 µm/L

BAP + 2.28 µm/L IAA. To select the media, we used the PGRs according to previous studies on Apiaceae family by changing the PGRs concentrations²⁸⁻³⁰. These media were selected as a superior environment for callus induction and indirect regeneration of *Trachyspermum ammi*. The optimized pH of the media was adjusted to 5.7-5.8 before the addition of 7% agar. All hormones were added to the media before autoclaving. Afterward, the culture media were autoclaved at 121°C and 1.2 kg cm⁻² pressure for 20 min.

Somatic embryogenesis induction

For the induction of somatic embryogenesis, the calluses were sub-cultured on the media with a low amount of plant growth regulators based on the following ratios: 1) MS + 4.5 µm/L 2,4-D + 2.32 µm/L kin; 2) MS + 4.5 µm/L 2,4-D + 0.09 µm/L kin; 3) B5 + 1.07 µm/L NAA + 0.89 µm/L BAP; 4) B5 + 0.54 µm/L NAA + 0.45 µm/L BAP + 1.14 µm/L IAA. The percentage of embryogenic calluses was calculated in each Petri dish.

Plant regeneration and acclimatization

To regenerate the plantlets, 3-week embryogenic calluses (MS) and 4-week embryogenic calluses (B5) were subcultured on MS medium supplemented with 0.93 µm/L kin. In order to study seedlings and their exposure to greenhouse conditions, the plantlets were subcultured on MS medium without PGRs for 10 days. The roots of the plantlets were gently washed with tap water to remove agar. Afterward, the seedlings were transferred to plastic pots containing sterilized soil (vermiculite, garden soil and perlite). At this stage, it is important to maintain the moisture of the seedlings. Hence, they were covered with a plastic cover. After two weeks, the seedlings were cultured into larger pots and eventually transferred to the greenhouse.

Statistical analysis

The treatments were tested in factorial experimental based on Completely Randomized Design (CRD) with four replicates (Petri dish) and 18 explants. In addition, the following traits were studied: callus induction, the number of days required for the appearance of callus, embryogenic callus and regeneration. Since some of the data were not normally distributed, data conversion (exponential) was applied. The mean values were compared, using Duncan's multiple range test at 5% probability level. Kruskal-Wallis test with a completely randomized design was used for the data that were not normalized.

The data were statistically analyzed, using SPSS (version 21), SAS (version 9.3) and MINITAB (version 16).

Results

Callus induction and embryogenic calluses were observed in all explants. The regeneration occurred only in hypocotyl and epicotyl explants (Fig. 1). The best treatment for callus induction was MS medium supplemented with 8.92 µm/L 2,4-D + 2.32 µm/L kin (hypocotyl). Additionally, the best medium was B5 medium supplemented with 1.07 µm/L NAA and 0.89 µm/L BAP (epicotyl). There were large callus in MS and B5 media for all three ecotypes. The largest callus induction was observed in hypocotyl of Qom and Yazd ecotypes on MS medium and hypocotyl of Qom, Yazd and Rafsanjan ecotypes on B5 medium; however, the lowest callus induction was in the node of Rafsanjan ecotype on MS medium. (Fig. 2). Comparison of callus induction between the two media indicated that B5 medium had a high percentage of callus induction for explants and ecotypes (Fig. 2).

After 30 to 35 days and depending on the explants and ecotypes, the calluses were sub-cultured on MS medium with less than 2,4-D and kinetin for induction of embryogenesis. The best treatment for embryogenic calluses was MS medium supplemented with 4.5 µm/L 2,4-D and 2.32 µm/L Kin. On B5 medium after a month, the calluses were subcultured on the same medium with constant PGRs, and then the embryogenic calluses emerged. The bright yellow and brittle calluses such as embryogenic calluses were compared with green and juicy calluses such as non-embryogenic calluses. The largest embryogenic calluses were observed in hypocotyl of Qom and Yazd ecotypes on MS medium and epicotyl of Qom

ecotype on B5 medium, while the lowest calluses were observed in the node of Rafsanjan and Yazd ecotypes on MS medium (Fig. 3). Comparison of the percentage of embryogenic calluses between both media revealed that B5 medium had a higher

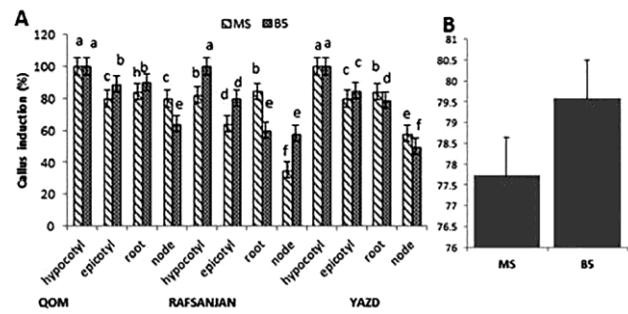


Fig. 2 — (A) Callus induction of different ecotypes and explants on MS and B5 media supplemented with plant regulators after 18-25 days. Different letters indicate a significant difference between treatments at $P \leq 0.05$ according to the Duncan test; And (B) Mean comparison of the percentage of callus induction on MS and B5 media for all ecotypes (Qom, Yazd and Rafsanjan) and explants (hypocotyl, epicotyl, root and node)

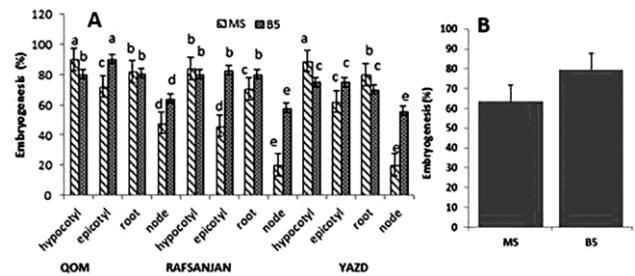


Fig. 3 — (A) Embryogenic calluses induction of different ecotypes and explants on MS and B5 media supplemented with plant regulators after 45-50 days. Different letters indicate a significant difference between treatments at $P \leq 0.05$ according to the Duncan test; and (B) Mean comparison of the percentage of embryogenic calluses on MS and B5 media for all ecotypes (Qom, Yazd and Rafsanjan) and explants (hypocotyl, epicotyl, root and node)

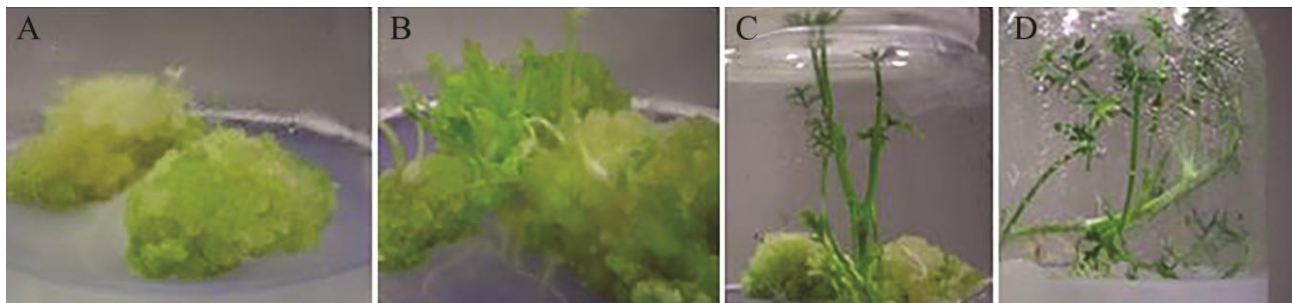


Fig. 1 — *In vitro* plant regeneration of *Trachyspermum ammi* (A) Callus induction from hypocotyl explants on MS medium; (B) Leaf and root formation from somatic embryogenic callus on MS medium; (C & D) Regenerated plant on MS medium; (E) Callus induction from epicotyl explants on B5 medium; (F) Leaf formation from somatic embryogenic callus on B5 medium; and (G & H) Regenerated plant on MS medium

percentage than MS medium for explants and ecotypes (Fig. 3).

The embryogenic calluses were subcultured on MS medium supplemented with 0.93 $\mu\text{m/L}$ Kin on MS medium after two months. Moreover, the regeneration of hypocotyl explants was observed in three ecotypes (Qom 83%, Rafsanjan 35%, and Yazd 45%) (Fig. 4). This regeneration was simultaneous with the appearance of shoot and root for hypocotyl explants on MS medium. Different appearances of ecotypes to be regenerated can be due to different expression genes or groups of genes in these ecotypes. On B5 medium after 70 days, the embryogenic calluses were transferred to the MS medium supplemented with 0.93 $\mu\text{m/L}$ Kin. The shoot regeneration of epicotyl explants was observed in three ecotypes (Qom 38.4%, Rafsanjan 20.1%, and Yazd 23.51%); also, the shoot regeneration was in the node of Qom (Fig. 4). Subsequently, the plantlets were transferred to MS medium supplemented with 0.57 $\mu\text{m/L}$ IAA, and they showed root formation for epicotyl explants. Comparison of regeneration percentage between the two media demonstrated that MS medium had a higher percentage of regeneration than B5 medium for explants and ecotypes (Fig. 4). Furthermore, comparison of the regeneration percentage between the three ecotypes indicated that Qom ecotype had the highest percentage of regeneration (Fig. 4).

According to these results, 2,4-D is not necessary on MS medium to regenerate hypocotyl explants. Regeneration was not observed on MS medium supplemented with 4.5 $\mu\text{m/L}$ 2,4-D + 0.09 $\mu\text{m/L}$ kin.

Therefore, regeneration failed in the presence of low kinetin or the absence of kinetin. The highest regeneration was obtained on B5 medium from epicotyl explant of Qom ecotype. In this medium, regeneration was not observed on B5 medium supplemented with 0.54 $\mu\text{m/L}$ NAA + 0.45 $\mu\text{m/L}$ BAP + 1.14 $\mu\text{m/L}$ IAA. Thus, regeneration failed in the presence of low BAP and high IAA. Furthermore, auxin (IAA) was essential in rooting the regenerated plantlet. Indirect regeneration occurred during days 85-90 depending on the ecotypes.

Results of Kruskal–Wallis test, for the number of days required for callus appearance on MS medium (MS + 8.92 $\mu\text{m/L}$ 2,4-D + 2.32 $\mu\text{m/L}$ Kin) showed that epicotyl explants, node, root, and hypocotyl have, respectively, the highest upper-median and number of days for callus appearance (Table 1). In contrast, it was found that the ecotype of Qom had the lowest

Table 1 — Kruskal–Wallis test for days required for callus initiation in 4 different explants and 4 media

| | | Media* | | | | | | | |
|------------|-----------|------------|-----------|------------|-----------|------------|-----------|-----------|-----------|
| | | 1 | | 2 | | 3 | | 4 | |
| Ex-plants | Mean Rank | Ex-plants | Mean Rank | Ex-plants | Mean Rank | Ex-plants | Mean Rank | Ex-plants | Mean Rank |
| Hypo cotyl | 16.83 | Hypo cotyl | 21.17 | Hypo cotyl | 12.50 | Hypo cotyl | 16.38 | | |
| epicotyl | 34.50 | epicotyl | 39.83 | epicotyl | 23.50 | epicotyl | 41.50 | | |
| root | 19.83 | root | 15.83 | root | 20.50 | root | 18.83 | | |
| node | 23.83 | node | 21.17 | node | 19.50 | node | 21.73 | | |

[* (1) MS + 8.92 $\mu\text{m/L}$ 2,4-D + 2.32 $\mu\text{m/L}$ kin; (2) MS + 4.5 $\mu\text{m/L}$ 2,4-D + 0.5 $\mu\text{m/L}$ kin; (3) B5 + 1.07 $\mu\text{m/L}$ NAA + 0.89 $\mu\text{m/L}$ BAP; and (4) B5 + 1.07 $\mu\text{m/L}$ NAA + 0.45 $\mu\text{m/L}$ BAP + 2.28 $\mu\text{m/L}$ IAA]

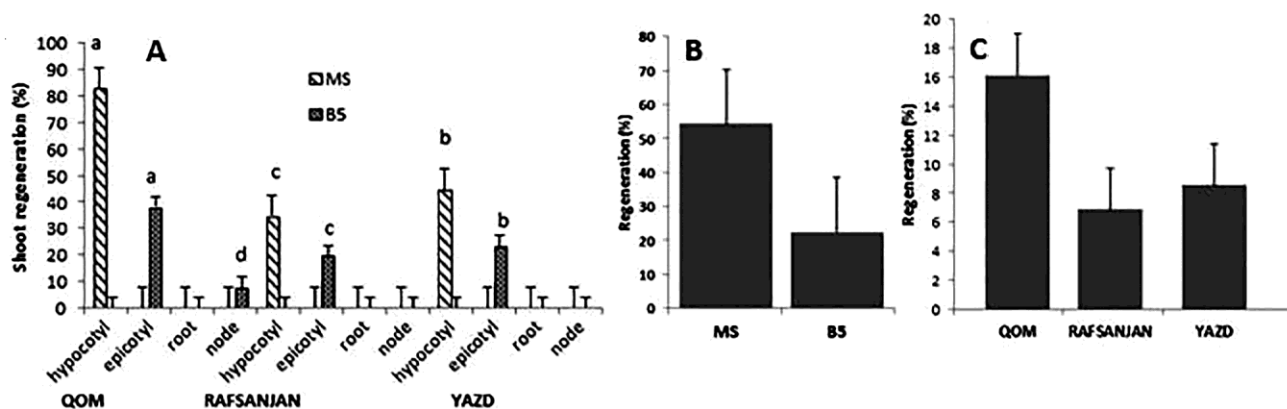


Fig. 4 — (A) Shoot regeneration of different ecotypes (Qom, Shahedieh Yazd, Rafsanjan) and explants (hypocotyl, epicotyl, root and node) on MS and B5 media supplemented with plant regulators. Different letters indicate a significant difference between treatments at $P \leq 0.05$ according to Duncan test; (B) Mean comparison of the percentage of regeneration on MS and B5 media for all ecotypes (Qom, Shahedieh Yazd and Rafsanjan) and explants (hypocotyl, epicotyl, root and node); and (C): Mean Comparison of the percentage of Regeneration of 3 ecotypes (Qom, Shahedieh Yazd, Rafsanjan) on two media (MS and B5) and all explants (hypocotyl, epicotyl, root, and node)

Table 2 — Kruskal-Wallis test for days required for callus initiation in 3 different ecotypes and 4 media

| Eco-types | Media* | | | | | | | |
|------------|-----------|------------|-----------|------------|-----------|------------|-----------|--|
| | 1 | | 2 | | 3 | | 4 | |
| | Mean Rank | Eco-types | Mean Rank | Eco-types | Mean Rank | Eco-types | Mean Rank | |
| Qom | 13.50 | Qom | 14 | Qom | 14.50 | Qom | 18 | |
| Yazd | 40.50 | Yazd | 28.50 | Yazd | 21.50 | Yazd | 23 | |
| Raf-sanjan | 19.50 | Raf-sanjan | 31 | Raf-sanjan | 37.50 | Raf-sanjan | 32.50 | |

[* (1) MS + 8.92 $\mu\text{m/L}$ 2,4-D + 2.32 $\mu\text{m/L}$ kin; (2) MS + 4.5 $\mu\text{m/L}$ 2,4-D + 0.5 $\mu\text{m/L}$ kin; (3) B5 + 1.07 $\mu\text{m/L}$ NAA + 0.89 $\mu\text{m/L}$ BAP; and (4) B5 + 1.07 $\mu\text{m/L}$ NAA + 0.45 $\mu\text{m/L}$ BAP + 2.28 $\mu\text{m/L}$ IAA]

number of days required for callus appearance, followed by Rafsanjan and Yazd (Table 2). In addition, this test indicated the number of days required for callus appearance on B5 medium (B5 + 1.07 $\mu\text{m/L}$ NAA + 0.89 $\mu\text{m/L}$ BAP) and revealed that epicotyl, node, root, and hypocotyl had higher upper-median and number of days needed for callus appearance, respectively (Table 1). Moreover, Qom had the lowest number of days required for callus formation, while Yazd, and finally, Rafsanjan had the highest number of days for callus appearance (Table 2). In general, in both media, the highest number of days necessary for appearance of callus induction was for epicotyl and the lowest for hypocotyl explants.

Discussion

In vitro propagation is useful for reproducing, maintaining, and saving medicinal plants that are difficult to be regenerated by conventional methods. In this research, we reported indirect regeneration and somatic embryogenesis of the Iranian ecotypes of *Trachyspermum ammi* using explants of hypocotyl and epicotyl. The results of three ecotypes with four explants manifested that all calluses emerged, and the majority of callus induction belonged to hypocotyl in MS and epicotyl in B5 media. The highest callus induction was in media MS + 8.92 $\mu\text{m/L}$ 2,4-D + 2.32 $\mu\text{m/L}$ Kin and B5 + 1.07 $\mu\text{m/L}$ NAA + 0.89 $\mu\text{m/L}$ BAP. Yang *et al.*³¹ showed that 6-BA and 2,4-D concentrations significantly affected the induction frequency of embryogenic callus. Bayarmaa³² reported that the embryogenic callus in *Zygophyllum potaninii* was achieved from the medium supplemented with 2,4-D. The embryogenic calluses appeared with a gradual reduction of hormones in the majority of explants. Merkle & Nairn³³ demonstrated that somatic embryogenesis occurred in the medium with high concentration of auxin or in medium

containing lower concentration of auxin. Pais³⁴ reported that high yields of somatic embryos simplify the use of these embryos for the achievement of large numbers of plants and its use in crop improvement. Somatic embryogenesis is an impressive alternative procedure to increase the rapidity of plant proliferation. Plant propagation by somatic embryogenesis has numerous utilizations in genetic improvement of any plant species³⁵.

Embryogenic calluses from the hypocotyl explants on MS medium after sub-culturing it with (0.93 $\mu\text{m/L}$) kin and free 2,4-D showed a good regeneration in *Trachyspermum ammi* after 80 days. Zhao *et al.*³⁶ reported that cytokinin, in combination with auxin, plays an important role in starting somatic embryogenesis in cereals. Schaller *et al.*³⁷ declared that the interaction of auxin and cytokinin in growth and developmental processes of many plants has the utmost importance. Sedaghaty *et al.*³⁸ illustrated that kinetin inhibits the formation of somatic embryos and has negative effect on plant regeneration.

In our study, calluses on medium B5 + 1.07 $\mu\text{m/L}$ NAA + 0.89 $\mu\text{m/L}$ BAP with explants of epicotyl showed shoot regeneration after subculture to the MS medium supplemented with kin (0.93 $\mu\text{m/L}$) after 85 days. On this medium, the growth of calluses was very quick and with a large size. There are also some reports about regeneration of NAA and BAP in callus induction and regeneration of medicinal plants^{39,40}.

In this research, for the first time, we found that in ecotypes of *Trachyspermum ammi* with a low amount of thymol⁴¹, callus induction was very difficult, and there was very little regeneration (data not shown). However, in ecotypes with high levels of thymol, high callus induction was observed and relatively good regeneration in hypocotyl and epicotyl explants. Kumari *et al.*⁴² showed the growth-promoting effect of thymol nanoemulsion on soybean plant growth. Also, by reducing the amount of 2,4-D, embryogenic callus was induced, and by removing 2,4-D, regeneration occurred.

Conclusion

A reliable *in vitro* regeneration of *Trachyspermum ammi* was presented in the current study. The results demonstrated that hypocotyl and epicotyl explants with appropriate plant growth regulator can produce successful regeneration of *T. ammi*. Regenerating of *Trachyspermum Ammi* was acquired from hypocotyl and epicotyl explants during 80 - 90 days. The highest

indirect regeneration (83%) was observed in hypocotyl explants on MS medium supplemented with kinetin. These methods can have important roles in gene transformation and molecular research. They have some benefits such as higher regenerated plantlets, simultaneous shoot and root induction, and reduction of subcultures.

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Conflict of interest

The authors declare no conflicts of interest.

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